Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Antibacterial Effect of Essential Oils Encapsulated in Chitosan Nanoparticles Against *Staphylococcus aureus* Isolated From Minced Meat Retailed in Kafr Elshiekh City, Egypt

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Abstract

THE OBJECTIVE of the current investigation was to develop chitosan nanoemulsions obtained from two essential oils, namely, lavender and lemongrass. Besides, investigation of the prevalence of *Staphylococcus aureus* (*S. aureus*) in four kinds of the retailed minced meat (beef mince, camel mince, mutton mince, and chicken mince) (20 of each, 100 g) was investigated. In addition, the anti-*S. aureus* activities of the prepared nano emulsions were screened. The obtained results revealed the success to develop nanoemulsions from lavender and lemongrass essential oils. *S. aureus* was isolated from the examined meat mince at 60%, 50%, 30%, and 25% in the examined beef mince, camel mince, mutton mince, and chicken mince, respectively. The recovered *S. aureus* isolates had the ability to produce enterotoxins. In an experimental study, the produced lavender and lemongrass nanoemulsions had significant anti-*S. aureus* activities using beef mince as a substrate. Besides, they can extend the shelf life of the chilled mince to the 8th day as they were compared to the control which was spoiled after 4 days of chilling. In conclusion, the obtained results of the current study suggest the use of lavender and lemongrass nanoemulsions as food additive to extend the shelf life of the meat protects and to reduce the microbial counts.

Keywords: Lavender; lemongrass; nanoemulsions; *S. aureus*; minced meat.

Introduction

Recently, there has been a growing focus on the research and utilization of biodegradable and biocompatible polymer materials. This is due to concerns about the adverse environmental impacts which were associated with the use of plastic compounds [1, 2]. Chitosan has been thoroughly examined for its potential to produce active pure or composite films and coatings due to its suitable properties, such as inherent antibacterial efficacy and favorable physicomechanical qualities [3]. According to Ojagh *et al*. [4], chitosan is a straight molecule that is made up of β-(1–4)-2-acetamido-D-glucose and β-(1–4)-2-amino-D-glucose units. The material possesses favorable characteristics, particularly in terms of its capacity to interact well with living organisms and its propensity to break down naturally over time [5]. Therefore, it is regarded as a highly significant molecule with enormous potential for packaging various sorts of foods [6]. Several researchers have investigated the increase in antibacterial, antioxidant, physical, and mechanical properties of biodegradable films and coatings with the addition of essential oils (EOs) and extracts [7, 8].

An intriguing natural substance with the ability to augment the synergistic effects of certain antiseptics and antimicrobials is lavender essential oil (LEO), which is extracted from the blossoming tops of Lavandula angustifolia Mill (Lamiaceae). LEO is a fragrance component in the cosmetics business and has several uses in pharmaceutical products [9]. It

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(Received 16 October 2024, accepted 06 January 2025)
DOI: 10.21608/EJVS.2025.328966.2431

has been shown that LEO has beneficial effects on wound healing through immunomodulation [10]. Several pharmacological actions of this oil have been reported in the literature, including its antibacterial, antifungal, antioxidant, anxiolytic, anticonvulsant, and anticholinesterase properties [11, 12]. The FDA has designated LEO as Generally Recognized as Safe (GRAS). Our understanding of LEO's exact mode of action is still limited. Bacterial cell susceptibility may be changed because it influences the ultrastructure of the bacterial wall.

A coarse grass with a strong lemon flavor, Cymbopogon citratus (DC.) is also known as lemongrass and it is one of several species in the genus Cymbopogon. There are two species of lemongrass, Cymbopogon citratus (DC.) and East Indian Cymbopogon flexuosus (DC.), which are both perennial herbs that are extensively cultivated in tropical and subtropical countries. Cymbopogon citratus (DC) has a long history of medicinal use in various countries. Traditional medicines have made use of lemongrass to treat a variety of ailments, including coughs, tuberculosis, elephantiasis, malaria, ophthalmia, pneumonia, and vascular issues. Scientific studies have shown that lemongrass has a number of beneficial benefits, including calming, nervine, antiseptic, astringent, bactericidal, fungicidal, antioxidant, and antidepressant. Lemongrass oil has been shown in multiple tests to effectively combat bacteria, yeast, and fungi, both Gram-positive and Gram-negative strains [13].

The detection of particular microorganisms, such as *Staphylococcus aureus (S. aureus*) in food indicates that the food handlers have neglected to follow proper hygiene standards. The primary factors contributing to the contamination of minced meat are inadequate personal hygiene, insufficient sanitation of storage and preparation environments, and filthy utensils. Furthermore, the improper handling of meat is regarded as the primary origin of *S. aureus* which is commonly seen in the respiratory tract, skin, and wounds [14, 15].

Staphylococcus aureus is a Gram-positive bacteria that is pathogenic and can cause various zoonotic diseases and food poisoning [16]. Global occurrences of foodborne illnesses resulting from *S. aureus* and its enterotoxins have been documented internationally [15, 17, 18].

S. aureus is responsible for several disorders such as SFP, toxic shock syndrome, bacteremia, pneumonia, and soft tissue infections. The pathogenicity of *S. aureus* is enhanced through the action of many virulence factors, such as the genes that code for staphylococcal enterotoxins (SEs) [15]. There has been less focus on the potential of minced meat as sources of *S. aureus* and SEs.

Insight of the previous facts, this study is aimed at investigation of the prevalence of *S. aureus* in four types of minced meat including beef mince, camel mince, mutton mince, and chicken mince. In addition, the ability of the recovered *S. aureus* isolates to produce different kinds of enterotoxins was also screened. Besides, nano-emulsions were prepared from lavender and lemongrass oils. The anti-*S. aureus* potentials for the prepared nano-emulsions were also investigated.

Material and Methods

Chemicals and Nano emulsion preparation:

Lavender and lemon grass oils were provided from oils extract unit of Animal Health Research Institute (AHRI), Dokki, Giza. Tween 80 and glacial acetic acid were obtained from the Sigma-Aldrich Co.

Powdered chitosan (CS) with a deacetylation degree (DDA) of 93% was procured from Oxford Lab. Chem. El-Gomhoria for chemicals Co. of Egypt supplied the food grade sodium tripolyphosphate, which has a purity level of 99.5%.

The Nanomaterials Research and Synthesis Unit at AHRI was responsible for producing the two nanoemulsions that contained 1% chitosan. The oils and tween 80 were blended for 5 minutes in a homogeneous blender with 1000 watts of power. Following the procedure outlined by Bharmoria *et al.* [19], distilled water and 1% chitosan were gradually added to the oil mixture.

Preparation of Chitosan Nanoparticles:

Ionic gelation of chitosan and sodium tripolyphosphate (TPP) was used to create nanoparticles [20]. The presence of 1% acetic acid in chitosan and 0.7 milligrams per milliliter of TPP in water produced nanoparticles on their own. The ratio of TTP to chitosan was 1:3, and the mixture was stirred at room temperature for 1 hour.

Characterization of nanoemulsions and chitosan nanoparticle:

Using a JEM 1400F HRTEM (Japan) with beam energy of 300 keV, the two nanoemulsions were characterized according to the standards set by the field. Nanoemulsion electrical conductivity, surface charge (zeta potential), droplet size, and size distribution (PDI) were measured using the NANOTRAC-WAVE II Zetasizer (MICROTRAC, USA).

Bacteriological examination:

Minced meat collection

Minced meat from cattle, camel, mutton, and chicken (20 of each, 100 g) were collected for surveillance study from retail markets and butcher shops at different hygienic levels in Kafr Elshiekh city, Egypt. Samples were transferred to the laboratory without delay for bacteriological examination.

Sample preparation according to ISO (6888-1:2021)

To be exact, 25 g of minced meat, together with 225 ml of sterile peptone water (0.1%), was mixed well for 1.5 minutes in a sterile blender. Afterwards, serial dilutions were made ten times [21].

Isolation and identification of S. aureus

One milliliter of each of the prepared serial dilutions was equally dispersed onto agar plates made of Baired Parker medium (Oxoid, UK) using a sterile bent glass spreader. After 24 hours, the plates were transferred to an incubator and kept at 37 °C. Glossy and dark in hue, the colonies were tallied. Black, shiny, round, smooth, convex, with a short white border colonies were detected, which were presumed to be *S. aureus*. A zone of transparency stretched into the otherwise opaque material, around them. This was done by counting the colonies and then calculating the number of *S. aureus* per gram [22].

S. aureus was identified through a series of examinations

First, a morphological examination was conducted [23], followed by biochemical identification [24]. The bacterium was then subjected to various tests including catalase activity, oxidase activity, mannitol fermentation, growth in the presence of 10% NaCl, bile esculent test, detection of hemolysis, coagulase test, thermostable nuclease test (D-Nase activity), detection of Arginine decarboxylase (ADH), and fermentation of sugars [25].

Examination of S. aureus isolates for enterotoxin production

S. aureus strains were cultivated in tryptone soya broth supplemented with 5% sodium chloride using an orbital shaker (Lab-Line Instruments, Melrose Park, Calif.) set to 200 rpm. The cultures were incubated at 37 °C for 24 hours. Following the growth phase, the culture underwent centrifugation at 900 rpm for 20 minutes. The resulting supernatant was examined to determine the presence of SEs. The presence of enterotoxins was identified using the commercially available ELISA kits, following the directions which were provided by the manufacturer. Concisely, latex reagents that have been sensitized with antisera are combined with diluted supernatant and left to incubate overnight to detect SEA, SEB, SEC, and SED [26].

Anti-S. aureus activities of the tested nanoemulsions:

An experimental study was conducted to evaluate the anti-*S. aureus* of the prepared nanoemulsions as following. From beef mince, 6500 g was purchased from the same butcher shop and beef mince was artificially inoculated with *S. aureus* strain recovered from the present study at a concentration of 6 log cfu/g. The beef mince was then formulated into cubes each is weighting 25 g and assigned into 9 groups each contains 25 cubes for the planned 5 time periods (2, 2, 4, 6, and 8 days) of chilling at 4 °C. Each treatment was done by immersion for 30 min in the prepared emulsion just before the preservation. The groups were assigned as following. G1 left as a control with no treatment. G2 was exposed to Lavender oil 1%, G3 was exposed to lemongrass oil 1%, while G4 was exposed to chitosan 1%. G5 was exposed to Lavender nanoemulsion 0.5%, G6 was exposed to Lavender nanoemulsion 1%, G7 was exposed to lemongrass nanoemulsion 0.5%, G8 was exposed to lemongrass nanoemulsion 1%, while G9 was exposed to a mixture of lavender and lemongrass nanoemulsions 1%. *S. aureus* re-isolation was conducted as previously mentioned. The sensory attributes and rates of reduction were assessed based on the methodology outlined by Bourdoux *et al.* [27].

Statistical analysis:

S. aureus counts were transferred to log 10 cfu/g. Data were expressed as means ± SE. The collected results were evaluated statistically using the Analysis of Variance (ANOVA) test, as described before [28] followed by Tukey's Kramer HSD post hock test, where $P< 0.0$ is considered to be significant.

Results

The obtained results of the present study revealed that the generated lavender nanoemulsion had a small size distribution (poly dispersity index, or PDI) of 1.7 and an average particle size of 7.14 nm, according to Zetasizer data. The produced NPs have a surface charge of -14.5 mV. With a polydispersity index (PDI) of 0.3, the lemongrass nanoemulsion had small size dispersion with an average particle size of 8.55 nm. The generated NPs have a surface charge of -6.9 mV. Zetasizer data, on the other hand, revealed that chitosan 1% nanoparticles have an average size of 21.66 nm and a very narrow size distribution (PDI = 0.005). A surface charge of +200 mV was observed on the synthetic NPs (Fig. 1).

In this investigation, *S. aureus,* a significant foodborne pathogen, was detected in 60% of the beef mince, 50% of the camel mince, 30% of the mutton mince, and 25% of the chicken mince samples, respectively **(**Fig. 2). The highest overall *S. aureus* counts (3.69±0.31 log 10 cfu/g) were found in beef mince, followed by camel mince (3.59±0.25 log 10 cfu/g), mutton mince (3.06±0.33 log 10 cfu/g), and chicken mince (2.73±0.22 log 10 cfu/g), according to Table 1. The samples that were analyzed showed levels of SEs at 25%, 20%, 10%, and 10%,

respectively, when the present study was extended to detect enterotoxin formation (Fig. 3).

Nanoemulsions could achieve significant reduction in *S. aureus* counts as they were compared with the ordinary oils with no alteration in the sensory characteristics of the minced meat (Table 2).

Discussion

Nanotechnology has the potential to revolutionize food technology and safety in different ways. It could make it easier to track and trace contaminants, improve food storage techniques, increase the shelf life of food products, and even make it easier to incorporate health supplements or antibacterial agents into food. Thus, nanotechnology greatly improves the field of food science. In addition, nanotechnology is a new and exciting development that has the potential to revolutionize the food industry and conventional food science. A prime example of nanotechnology's impact on food systems is its use in processing and packaging. Nanoparticles can be prepared in a variety of ways to give them different physical properties, which opens up new possibilities for their use in food [29]. The generated NPs had comparable sizes to previous preparations of nanoparticles [30]. Also, in the past, nanochitosan films were made by mixing essential oil of *Mentha spicata* with methanolic extracts of grape seed and pomegranate peel [2].

The protein, amino acids, and minerals that were included in minced meat are of great quality. Nevertheless, microorganisms from the mincer or the hands of the operator can easily contaminate the beef product while it is being minced [14]. Consistent with the current study's findings, samples generated from various types of beef in Slovenia showed *S. aureus* contamination levels ranging from 10% to 78% [31]. Besides, minced meat sampled from hotels and supermarkets in Ethiopia was found contaminated with *S. aureus* at 12.1% [32]. In Saudi Arabia, minced meat was found contaminated with *S. aureus* at 38% and contained all types of the classical enterotoxins [33]. In Egypt, *S. aureus* was isolated from beef mince at comparable counts in samples collected from Zagazig city, Egypt [34]. Chicken meat products that were collected from Cairo city was also contained *S. aureus* at similar rates and the samples were found contaminated with SEs [35].

Staphylococcal enterotoxins (SEs) are the primary causative agents of Staphylococcus foodborne intoxication and are produced by staphylococci that test positive for coagulase. SEs refers to gastrointestinal exotoxins, as stated by Argudín *et al*. [36]. The SEs remains viable in the digestive tract following ingestion by humans due to their ability to withstand high temperatures, proteolytic enzymes, and other environmental factors. The identification of SEs is a reliable technique for confirming outbreaks because of their consistent characteristics and the ability of strains to produce enterotoxins. More than 20 enterotoxins of *S. aureus* have been discovered. Consuming food that is contaminated with enterotoxigenic *S. aureus* can readily result in foodborne outbreaks, as the stable qualities of SEs and the low dosage needed might cause symptoms [37]. According to a study conducted by the Center for Disease Control and Prevention, there were 241,188 reported instances of Staphylococcus aureus food poisoning (SFP) in the United States from 2006 to 2008. This resulted in 1064 hospitalizations and 6 deaths [17]. In China, S. aureus was responsible for 53.7% of food poisoning cases in 2015. S. aureus food poisoning is characterized by a sudden and swift occurrence of symptoms such as nausea, vomiting, and abdominal cramps [18].

In the next part of the current investigation, we studied the anti-*S. aureus* activities of the prepared nanoemulsions that were made from lavender and lemongrass. Interestingly, the used nanoemulsions could reduce *S. aureus* counts in a concentration-dependent manner and extend the shelf life of the chilled meat to the 8th day as they were compared with the control which was spoiled on the 4th day. The present study's findings are in line with those of other reports that lavender oil has anti-*S. aureus* actions in vitro [38, 39]. There is still lack of information about the precise mechanism of action of lavender essential oil. Theoretically, it changes the susceptibility of complete bacterial cells by affecting the ultrastructure of the bacterial wall. Research has demonstrated that linalool and linalyl acetate made up the majority of lavender essential oil, accounting for 34.1% and 33.3% of the total, respectively. Lavandulyl acetate, (Z)-β-ocimene, (E)-β-ocimene, β-caryophyllene, 1,8-cineole, terpinene-4-ol, and myrcene accounted for 2.4% and 2.4%, respectively, according to the same source. Lemongrass oil, like lavender oil, has been found to have strong anti-*S. aureus* effects in clinical isolates [41]. The compounds found in lemongrass oil are monoterpenes. As a main ingredient, citral is a naturally occurring combination of geranial and neral, two isomeric acyclic monoterpene aldehydes. Citral isn't the only antimicrobial in lemongrass; the plant also includes myrcene, geraniol, and geranyl acetate [42].

Conclusion

This study demonstrated the ability to synthesize nanoemulsions for lavender and lemongrass oils which have clear anti-*S. aureus* activities and could extend the shelf life of the chilled minced meat which was used as a substrate. Therefore, such nanoemulsions can be used as friendly candidates to reduce microbial contamination and extend the shelf life of meat products.

Acknowledgments

We would like to thank Fish Processing and Biotechnology Department, Faculty of Aquatic and Fisheries Sciences, Kafr Elsheikh University, Kafr Elsheikh, Egypt for their technical support.

Funding statement

This study didn't receive any funding support.

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study was done according to the ethical guidelines of Kafr Elsheikh University, Egypt.

Authors' contributions

All authors contributed equally to this study.

TABLE 1. *S. aureus* counts (log10/g) in the examined minced meat samples (n=20 for each).

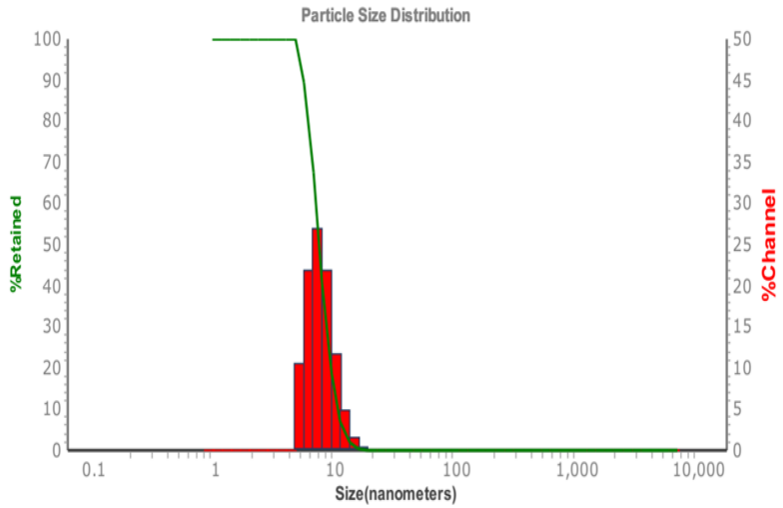
Animal	Minimum	Maximum	Mean ± SE
Beef mince	2.60	4.69	3.69 ± 0.31[a]
Camel mince	2.47	4.00	3.59 ± 0.25[a]
Mutton mince	2.00	3.90	3.06 ± 0.33[b]
Chicken mince	2.00	3.77	2.73 ± 0.22[b]

S.E :Standard error of means
Means carrying different superscripts letters are significantly different (P<0.05).

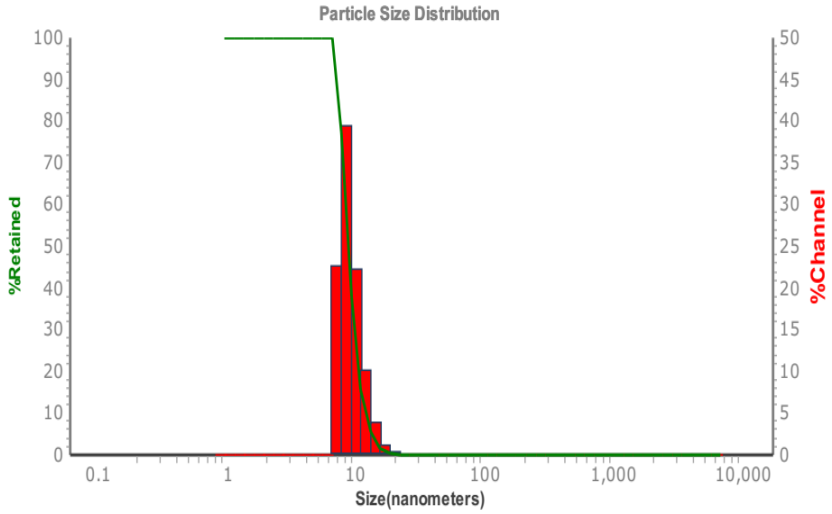
TABLE 2. Anti-*S. aureus* activities of the used nanoemulsions and essential oils.

	0	2	4	6	8
Control	6.70± 0.02a	7.22± 0.05a	8.56 ± 0.02a	S	S
Lav. oil 1%	6.35± 0.01a	5.66± 0.18b	5.18± 0.11 b	5.15± 0.11a	5.65± 0.13a
Lg. oil 1%	6.44± 0.12a	5.54± 0.02b	5.15± 0.12b	5.20 ± 0.12a	5.58± 0.12 a
Chitosan	6.55± 0.03a	5.61± 0.07b	5.48± 0.10 b	5.30± 0.01a	5.61± 0.08 a
Lav. 0.5 %	6.50± 0.43a	5.64± 0.18b	4.81± 0.06 c	4.93± 0.02 b	5.59± 0.52a
Lav. 1%	6.49± 0.19a	5.75± 0.019b	4.75± 0.01c	4.78± 0.05 c	4.84± 0.05b
Lg. 0.5%	6.42± 0.10a	4.54± 0.27c	4.34± 0.33d	4.70± 0.13 c	4.29 ± 0.22c
Lg. 1%	6.52± 0.17a	4.49± 0.17c	3.42 ± 0.04e	3.62± 0.01 e	3.43± 0.01d
Mix 1%	6.48± 0.11a	5.73± 0.04b	5.31± 0.15b	4.44 ± 0.07 d	4.69± 0.08 bc

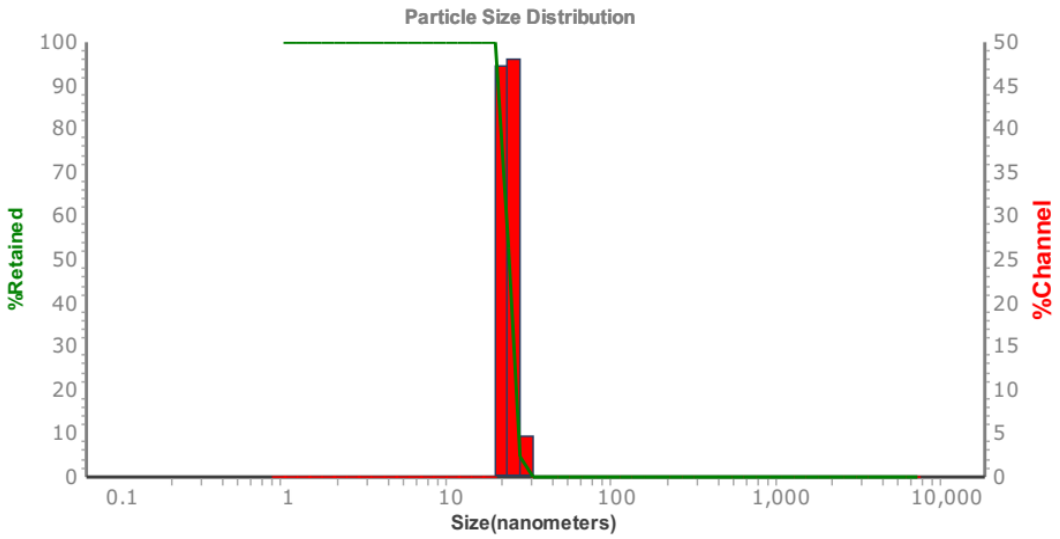
Lav stands for lavender, Lg refers to lemongrass, while S refers to spoiled. Values represent means ± SE (n =5) for each treatment at each time period (0, 2, 4, 6, and 8 days of preservation by chilling). Data within the same column carrying different superscript letters were considered to be statistically significant at P<0.05.



A



B



C

Fig. 1. Characterization of nano-emulsion (A) Lavender 20% nanoemulsion Particle size pattern of synthesized NPs showing size distribution of NPs of 206.4 nm. (B) lemongrass 20% nanoemulsion (C) chitosan 1% nanoparticle

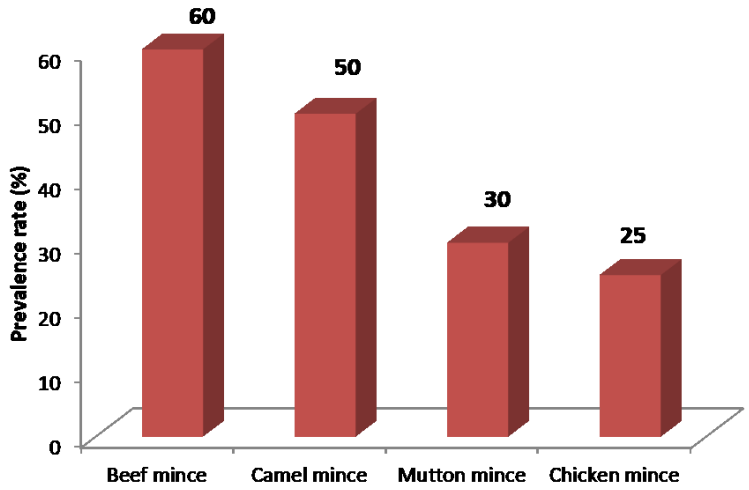


Fig. 2. Prevalence rates (%) of *S. aureus* in the examined minced meat samples (n = 20)

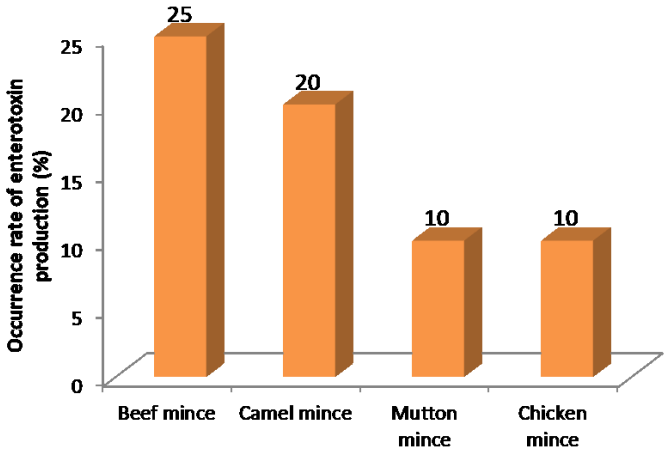


Fig. 3. Occurrence rate (%) of enterotoxin production among the recovered *S. aureus* isolates

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التأثير المضاد للبكتيريا للزيوت الأساسية المغلفة في جزيئات الكيتوزان ضد المكورات العنقودية الذهبية المعزولة من اللحم المفروم المباع في مدينة كفر الشيخ، مصر

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الملخص

كان الهدف من هذه الدراسة هو تطوير مستحلبات الكيتوزان الدقيقة و المستخلصة من زيتين أساسيين، وهما زيت اللافندر وزيت عشب الليمون. بالإضافة إلى ذلك، تم دراسة مدي انتشار المكورات العنقودية الذهبية في أربعة أنواع من اللحوم المفرومة المباعة بالتجزئة (لحم البقر المفروم، لحم الجمل المفروم، لحم الضأن المفروم، ولحم الدجاج المفروم) (20 من كل نوع، 100 جرام). بالإضافة إلى ذلك، تم فحص الأنشطة المضادة لبكتيريا المكورات العنقودية الذهبية لمستحلبات النانو المستخلصة. أظهرت النتائج التي تم الحصول عليها نجاح تطوير مستحلبات الكيتوزان الدقيقة و المستخلصة من زيت اللافندر وزيت عشب الليمون. تم عزل المكورات العنقودية الذهبية من اللحم المفروم الذي تم فحصه بنسبة 60%، 50%، 30%، و25% في اللحم البقري المفروم، لحم الإبل المفروم، لحم الضأن المفروم، ولحم الدجاج المفروم، على التوالي. كانت معزولات المكورات العنقودية الذهبية قادرة على إنتاج السموم المعوية. وفي دراسة تجريبية، كانت مستحلبات الكيتوزان الدقيقة و المستخلصة من زيت اللافندر وزيت عشب الليمون أنشطة مضادة لبكتيريا المكورات العنقودية الذهبية بشكل كبير باستخدام لحم البقر المفروم كركيزة. بالإضافة إلى ذلك، يمكن لتلك المستحلبات إطالة فترات حفظ اللحوم المفرومة المبردة إلى اليوم الثامن مقارنةً بالعينة الضابطة التي فسدت بعد 4 أيام من التبريد. في الختام، تشير النتائج التي تم الحصول عليها من الدراسة الحالية إلى امكانية استخدام مستحلبات الكيتوزان الدقيقة و المستخلصة من زيت اللافندر وزيت عشب الليمون كإضافات غذائية لتمديد فترة صلاحية اللحوم وتقليل عدد المكورات العنقودية الذهبية بها.

الكلمات الدالة: مستحلبات الكيتوزان الدقيقة ، اللافندر ، زيت عشب الليمون ؛ المكورات العنقودية الذهبية؛ اللحم المفروم